BBA 66703

PURIFICATION AND PROPERTIES OF THREONINE DEHYDRATASE FROM *RHODOPSEUDOMONAS SPHEROIDES*

G. J. BARRITT* AND J. F. MORRISON

Biochemistry Department, John Curtin School of Medical Research, Institute of Advanced Studies, Australian National University, Canberra, A.C.T. (Australia)

(Received April 13th, 1972)

SUMMARY

Biosynthetic threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16) has been purified about 3000-fold from cell-free extracts of the photosynthetic bacterium, *Rhodopseudomonas spheroides*. The addition of dithiothreitol, pyridoxal phosphate and allothreonine was essential for stabilization of the enzyme during both purification and storage. The molecular weight of the enzyme was estimated to be in the vicinity of 200 000.

Kinetic studies indicated that, at low concentrations of threonine, there is a time-dependent loss of enzymic activity which is influenced by the pH and composition of buffers, as well as by KCl and pyridoxal phosphate. In the presence of isoleucine, there is a relatively slow development of the steady-state inhibited rate. No lag period was associated with the inhibition of the reaction by higher concentrations of allothreonine or with the activation by lower concentrations of this compound. It has been concluded that the time-dependent inhibition by isoleucine can be accounted for on the assumption that this reactant induces the enzyme to undergo isomerization reactions which are slow relative to the rate of catalysis.

INTRODUCTION

It has been established that biosynthetic threonine dehydratase (L-threonine hydro-lyase (deaminating), EC 4.2.1.16), which catalyses the reaction

$$\text{Threonine} \xrightarrow{\text{Pyridoxal phosphate}} \alpha\text{-ketobutyrate} + NH_3,$$

is the first enzyme of the pathway that leads to the biosynthesis of isoleucine in a variety of microorganisms[1]. Further, it has been shown that the enzymes from such sources are inhibited by isoleucine and activated by valine[1]. In attempts to determine

Abbreviations: Bicine, *N,N*-bis(2-hydroxyethyl)glycine; TES, *N*-tris(hydroxymethyl)-methyl-2-aminoethane sulphonic acid; Tricine, *N*-tris(hydroxymethyl)methylglycine.

* Present address: Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106, U.S.A.

the mechanism(s) by which these modifiers exert their effects, kinetic investigations have been made with the pure enzymes from *Salmonella typhimurium*[2] and *Bacillus subtilis*[3,4], as well as less highly purified preparations from the non-sulphur, photosynthetic bacterium, *Rhodospeudomonas spheroides*[5], and other organisms[6–9]. However, some studies have been hampered by the instability of certain biosynthetic threonine dehydratases[5–9].

Because of the differences in the regulation of the biosynthesis of isoleucine, valine and amino acids of the aspartate family by photosynthetic and non-photosynthetic bacteria[10–12], it was of interest to pursue investigations of the effects of isoleucine and valine on the activity of threonine deaminase from *R. spheroides*. As a prelude to these studies, attention has been directed towards the elucidation of conditions which are required to produce a stable form of enzyme. This report describes the stabilization of threonine dehydratase from *R. spheroides* in the presence of dithiothreitol, pyridoxal phosphate and allothreonine, as well as the further purification and some general properties of the enzyme.

MATERIALS AND METHODS

Organism

The strain of *R. spheroides*, which came from the stock of Dr R. Y. Stanier, was obtained from Dr C. Appleby.

Chemicals

L-Threonine was supplied by the Cyclo Chemical Corporation; L-isoleucine, L-valine, *N*,*N*-bis(2-hydroxyethyl)glycine (Bicine), *N*-tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid (TES), *N*-tris(hydroxymethyl)methylglycine (Tricine), pyridoxal phosphate, dithiothreitol, lactate dehydrogenase (EC 1.1.1.27) and yeast alcohol dehydrogenase (EC 1.1.1.1) by Calbiochem; DL-*allo*threonine by the Pierce Chemical Company. α-Ketobutyrate and tris(hydroxymethyl)aminoethane (Tris) were purchased from the Sigma Chemical Company, and 2,4-dinitrophenylhydrazine (analytical grade) from British Drug Houses. *N*-Ethylmorpholine was the product of Eastman Organic Chemicals, and before use was distilled twice under reduced pressure[13]. Creatine kinase (EC 2.7.3.2) was prepared and stored as described by Morrison *et al.*[13]. All other reagents were commercial products of the highest grade available.

Buffer solutions

Buffer solutions had the following compositions: Buffer A, 0.05 M potassium phosphate–0.12 mM L-isoleucine (pH 8.1); Buffer B, 0.05 M *N*-ethylmorpholine·HCl–0.12 mM L-isoleucine (pH 8.0); Buffer C, 0.025 M *N*-ethylmorpholine·HCl–4.0 mM allothreonine–20 μM pyridoxal phosphate (pH 8.0); Buffer D, 0.05 M *N*-ethylmorpholine–HCl–0.5 M KCl–4.0 mM allothreonine–20 μM pyridoxal phosphate (pH 8.0); Buffer E, 0.1 M Bicine–NaOH (pH 8.1 at 55 °C)–0.5 M KCl–10.0 mM allothreonine–40 μM pyridoxal phosphate–2.0 mM isoleucine–1.0 mM dithiothreitol–0.5 mM EDTA; Buffer F, 0.025 M Bicine·NaOH–5.0 mM *allo*threonine–20 μM pyridoxal phosphate (pH 8.5); Buffer G, 0.05 M Bicine·NaOH–0.5 M KCl–5.0 mM allothreonine–20 μM pyridoxal phosphate (pH 8.0). With the exception of Buffer E, all buffer solutions also contained 0.1 mM dithiothreitol and 0.1 mM EDTA.

Growth of cells

Cells were grown under photosynthetic conditions in batches of 60 l (six 10-l glass bottles) in a malate–glutamate medium[5], supplemented with 12 μM ferric citrate. Preparation of the inoculum and inoculation of the medium were performed as described by Lascelles[14]. Illumination was provided by eight 150-W Phillips Comptalux flood lights, and the temperature was maintained at 30–35 °C by cooling the bottles with a fan[14]. After 24 h, the cells were harvested using a Sharples Super Centrifuge, washed in Buffer A, centrifuged, and stored at −15 °C. The average yield of cells was about 4.5 g (wet weight) per l of medium.

Fractionation with $(NH_4)_2SO_4$

The weight of solid $(NH_4)_2SO_4$ required to give the desired degree of saturation was calculated as described by Noltmann *et al.*[15].

Measurement of threonine dehydratase activity

Threonine dehydratase activity was measured by a modification of the method described by Datta[5]. Reaction mixtures contained, in a total volume of 1.0 ml: 0.1 M Bicine–NaOH (pH 8.0), 20 mM threonine and 10 μM pyridoxal phosphate. Tubes were equilibrated at 30 °C before the addition of an appropriate amount of enzyme from a Hamilton microsyringe. To ensure that initial velocities were being determined, reactions were run for four time periods, including a zero time control. They were stopped by the addition of 0.2 ml of 0.06% (w/v) 2,4-dinitrophenylhydrazine in 2 M HCl, except when larger amounts of protein were present. Under the latter conditions, stopping was done by the addition of 0.3 ml of 30% (w/v) trichloroacetic acid. The tubes were then cooled in ice and the precipitated protein removed by centrifugation before the addition of 0.2 ml of the dinitrophenylhydrazine reagent to samples (1.0 ml) of the supernatant solutions. Following the addition of dinitrophenylhydrazine, the tubes were allowed to stand 20 min. Then 0.8 ml of 2.5 M NaOH was added and after a further 20 min the absorbance was measured at 440 nm in a cell of 1-cm light path, using a Gilford 300 spectrophotometer. The extinction coefficient of the dinitrophenylhydrazone of α-ketobutyrate was determined to be 5200 $M^{-1} \cdot cm^{-1}$.

A unit of threonine dehydratase activity was taken to be the amount of enzyme which catalyses the formation of 1 μmole of α-ketobutyrate per h under the above conditions. Specific activity is defined as units per mg of protein.

Estimation of protein concentration

The concentration of protein was determined by either the biuret method[16] or spectrophotometrically at 260 and 280 nm[17], according to the purity of the enzyme.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed by the method of Orr[18] which utilises a discontinuous buffer system. This consisted of 38 mM TES–triethanolamine (pH 6.3)–1.0 mM EDTA in the cathode chamber and 32 mM phosphate–triethanolamine (pH 5.8)–1.0 mM EDTA in the anode chamber. Electrophoresis was carried out at 4 °C with a current of 2 mA per tube. The positions of protein bands were detected by staining the gel with Amido Black[19] while a modification of the procedure for the

detection of lactate dehydrogenase activity[20] was used to determine which protein bands possessed threonine dehydratase activity. The gels were incubated at 30 °C in a solution containing 0.1 M Bicine–NaOH (pH 8.0), 20 mM threonine, 10 μM pyridoxal phosphate, 0.15 mM NADH and lactate dehydrogenase (12 μg/ml). After 30 min the gels were removed, washed with distilled water and incubated in the dark for 1 h at 37 °C in a solution containing 0.1 M Bicine–NaOH (pH 8.0), nitrotetrazoleum blue (0.33 mg/ml) and phenazine methosulphate (20 μg/ml). Threonine deaminase activity was revealed as a purple band.

Estimation of molecular weight by chromatography

The molecular weight of threonine deaminase was determined by chromatographing samples of the enzyme and marker proteins on a column of Sephadex G-200 (0.9 cm × 30 cm) according to the procedure of Andrews[21]. The column was equilibrated with 0.1 M TES–NaOH (pH 7.4) containing 0.1 M KCl, 1.0 mM dithiothreitol, 0.1 M EDTA, 20 μM pyridoxal phosphate and 5.0 mM allothreonine. The same solution was used to elute the proteins. Elution volumes for catalase, yeast alcohol dehydrogenase, horse radish peroxidase and creatine kinase were obtained by measurement of their catalytic activities as described by Chance[22], Racker[23], Chance and Maehly[24] and Noda *et al.*[25], respectively, while that for cytochrome *c* was determined from absorption measurements at 412 nm[21].

Sucrose gradient centrifugation

Sucrose gradients were prepared in 0.1 M TES–NaOH (pH 7.4), containing 0.1 M KCl, 20 μM pyridoxal phosphate, 5.0 mM allothreonine, 1.0 mM dithiothreitol and 0.1 mM EDTA and centrifugation carried out by the method of Martin and Ames[26]. After purified threonine dehydratase (1600 units/mg, 70 μg) and yeast alcohol dehydrogenase had been layered on the top of the gradient, runs were made for 12 h at 4 °C and 38 000 rev./min using a SW-39 rotor in a Beckman L-2 ultracentrifuge.

RESULTS

Purification of threonine dehydratase

Threonine dehydratase was purified from cell-free extracts of *R. spheroides* by modifying and extending the procedure of Datta[5]. All steps were performed at 4 °C unless otherwise stated.

Preparation of crude extracts

Frozen cells (300 g) were thawed, suspended in 1.0 l of Buffer A and disrupted in a Ribi Cell Fractionator at 20 000 lb/inch^2. The resulting suspension was brought to pH 8.0 with NH_4OH (*d* 0.88) and the cell debris removed by centrifuging at 23 000 × *g* for 30 min. After readjustment to pH 8.0 with NH_4OH, the supernatant was centrifuged at 36 000 × *g* for 20 h.

Removal of nucleic acid

N-Ethylmorpholine–HCl buffer (0.5 M, pH 8.0) was added to the supernatant from the previous step to bring the final concentration of *N*-ethylmorpholine to 0.05 M. This was followed by the addition of isoleucine, dithiothreitol and EDTA so that

the final concentration of each compound was 0.1 mM. A solution of protamine sulphate (1%, w/v) in Buffer B was added with stirring over a period of 20 min after which the suspension was stirred for a further 10 min before centrifuging at 23 000 × *g* for 15 min. The amount of protamine sulphate required to precipitate nucleic acid was determined as described by Bucher[27].

$(NH_4)_2SO_4$ fractionation

The concentration of *N*-ethylmorpholine in the supernatant from the previous step was adjusted to 0.1 M with 0.5 M *N*-ethylmorpholine–HCl (pH 8.0) and pyridoxal phosphate added to a final concentration of 20 μM. The solution was then brought to 0.32 saturation by adding solid $(NH_4)_2SO_4$ slowly and with stirring. After stirring for a further 15 min, the precipitate was removed by centrifuging for 15 min at 23 000 × *g* and the supernatant brought to 0.48 saturation by the further addition of solid $(NH_4)_2SO_4$. The mixture was stirred for 15 min, centrifuged as described above and the precipitate suspended in 100 ml of Buffer B containing 20 μM pyridoxal phosphate. After adjustment of the concentration of protein to 15 mg/ml; of *N*-ethylmorpholine (pH 8.0) to 0.1 M and each of isoleucine, EDTA and dithiothreitol to 0.1 mM, the solution was again fractionated with solid $(NH_4)_2SO_4$. Fractions precipitating from 0.22–0.32 saturation and from 0.32–0.45 saturation were collected and each dissolved in 30 ml of Buffer B containing 20 μM pyridoxal phosphate. The two solutions were then combined and dialysed overnight against 1.0 l of Buffer C.

Chromatography on DEAE-cellulose

The dialysed solution of protein from the 0.22–0.32 and 0.32–0.45 $(NH_4)_2SO_4$ saturation fractions was applied to a column (4.5 cm × 25 cm) of DEAE-cellulose previously equilibrated with Buffer C. The enzyme was eluted with a linear gradient formed from 1200 ml of Buffer C and the same volume of Buffer C containing 0.5 M KCl. Fractions (11.4 ml) were collected and those with a specific activity greater than 35 were pooled and concentrated to 20 ml using a Diaflo ultrafiltration cell fitted with a UM-10 membrane. The elution profile for threonine dehydratase is illustrated in Fig. 1A.

Chromatography on Sephadex G-100

The concentrated solution was passed through a column (2.5 cm × 140 cm) of Sephadex G-100 equilibrated with Buffer D and the enzyme eluted with the same buffer. Fractions (5.7 ml) containing threonine dehydratase with a specific activity of 120 or greater were pooled and concentrated to about 12 ml by ultrafiltration (Fig. 1B).

Heat denaturation

After dialysis of the concentrated solution from the previous step against 300 ml of Buffer E for 6 h, it was heated with periodic agitation in a water bath at 55 °C for 12 min. The mixture was then cooled quickly in an ice bath and the denatured protein removed by centrifugation for 10 min at 36 000 × *g*.

Chromatography on DEAE-Sephadex

The above supernatant was dialysed overnight against 500 ml of Buffer F, con-

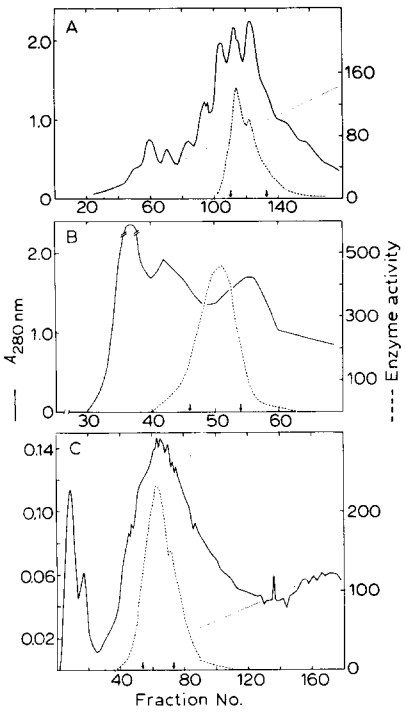


Fig. 1. Patterns for the elution of protein (———) and enzyme (— — —) from columns of DEAE-cellulose (A), Sephadex G-100 (B) and DEAE-Sephadex (C). The straight lines (A, C) represent the rate of increase in the concentrations of the eluting gradients which were formed as described in the text. The fractions taken for further purification were those between the arrows.

taining 0.2 M KCl, and applied to a column (2 cm × 30 cm) of DEAE-Sephadex A 50 equilibrated with the same buffer. The column was washed with 50 ml of buffer and the enzyme eluted with a linear gradient generated from 300 ml of Buffer F–0.2 M KCl and the same volume of Buffer F–0.27 M KCl (Fig. 1C). Those fractions (3.1 ml) which contained threonine dehydratase with a specific activity greater than 1000 μmoles/h per mg of protein were combined. Solid KCl was added to give a final concentration of 0.5 M and the volume reduced to about 4 ml by ultrafiltration before dialysis overnight against 300 ml of Buffer G.

A summary of the yields and specific activities of the various fractions obtained during the purification procedure are given in Table I. The specific activity of the purified enzyme was 2900 μmoles/h per mg of protein, compared with a value of 145 μmoles/h per mg of protein previously reported by Datta[5].

Properties of the purified enzyme

When the enzyme was stored at −15 °C in Buffer G at a concentration of 2.7 mg/ml, there was no detectable loss of activity over a period of twelve months. Only a small loss of activity (5% in 2 months) was observed when storage was done at 0 °C.

Polyacrylamide gel electrophoresis of the purified enzyme showed the presence of three major and two minor bands of protein (Fig. 2A). Of these, only the slowest moving of the major bands exhibited threonine dehydratase activity (Fig. 2B). When

TABLE I

SUMMARY OF YIELDS AND SPECIFIC ACTIVITIES OF FRACTIONS OBTAINED DURING THE PURIFICATION OF THREONINE DEHYDRATASE FROM *R. spheroides*

Wet weight of cells, 300 g.

Purification step	*Volume (ml)*	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg)*
Extract	1430	28 000	47 500	0.6
Supernatant after high speed centrifugation	1360	26 800	15 800	1.7
Supernatant after protamine sulphate treatment	1740	24 900	10 880	2.3
$(NH_4)_2SO_4$ precipitate (0.22–0.45 saturation)	36	26 500	1 612	16.4
Eluate from DEAE-cellulose column	19	21 900	386	56.6
Eluate from Sephadex G-100 column	12	17 000	83	205
Supernatant after heat treatment	12.8	15 900	22	730
Eluate from DEAE-Sephadex column	4.2	8 900	2.7	2900

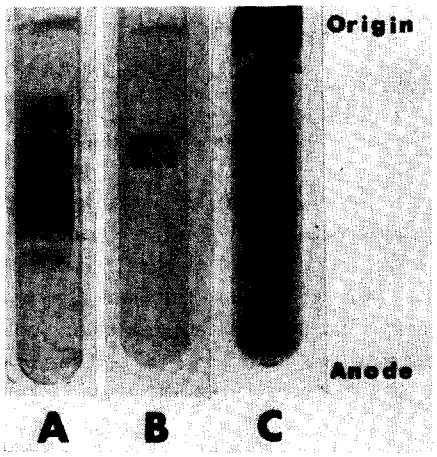


Fig. 2. Polyacrylamide gel electrophoresis of purified threonine dehydratase (150 μg; 1500 units per ml) before (A, B) and after treatment with 6 M urea for 1 h in the presence of 50 mM TES–NaOH (pH 7.4) containing 0.1 M mercaptoethanol (C). The positions of the bands of protein (A, C) and enzymic activity (B) were determined as described under Materials and Methods.

the enzyme preparation was treated with urea and mercaptoethanol prior to electrophoresis, there was obtained a single band of protein whose mobility corresponded to the fastest moving of the major bands observed with the native enzyme (Fig. 2C). These results suggest that the two dense bands of inactive protein observed after electrophoresis of the native enzyme (Fig. 2A) are degraded forms of threonine dehydratase, and that treatment with urea and mercaptoethanol promotes complete

degradation of the native enzyme to form a single protein species (Fig. 2C). The two major species of inactive protein (Fig. 2A) may either have arisen during electrophoresis, or have been present in the enzyme preparation prior to electrophoresis. Since some heterogeneity of the protein was observed when the purified enzyme was subjected to centrifugation in a sucrose gradient, it was concluded that at least a portion of the inactive protein (Fig. 2A) was present before electrophoresis. Thus it appears that the purified enzyme may contain degraded forms of threonine dehydratase which are inactive, as well as some foreign protein (represented by the two minor bands of Fig. 2A).

Molecular weight

The molecular weight of threonine dehydratase was determined by the method of Andrews[21] (Fig. 3), and from the results of two experiments a mean value of 210 000 ± 28 000 was calculated. It should be noted that the omission of allothreonine from the buffer solution (see Materials and Methods) or the replacement of

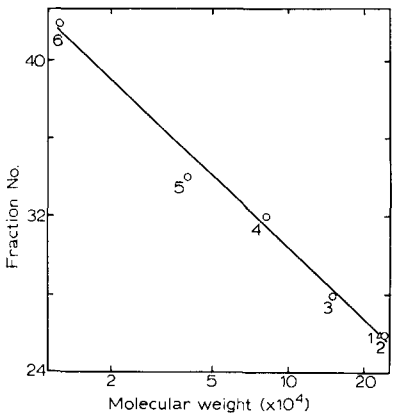


Fig. 3. Estimation of the molecular weight of threonine dehydratase by gel filtration. The proteins used (as well as their amounts and molecular weights) were: 1, threonine dehydratase (58 μg); 2, catalase (110 μg; 240 000); 3, yeast alcohol dehydrogenase (100 μg; 151 000); 4, creatine kinase (120 μg; 81 000); 5, horse radish peroxidase (90 μg; 40 000); 6, cytochrome *c* (100 μg; 12 000). Conditions were as described in Materials and Methods.

allothreonine by 1.0 mM isoleucine did not alter the molecular weight of the enzyme. The aforementioned value may be compared with a mean estimate of 165 000 ± 14 000 as obtained from two experiments using the sucrose gradient centrifugation technique of Martin and Ames[26] with yeast alcohol dehydrogenase[28] as a marker protein.

Stability of threonine dehydratase

When attempts were made to purify the enzyme by chromatography on Sephadex G-100 in the presence of 0.05 M *N*-ethylmorpholine–HCl (pH 8.0) and 0.6 mM L-isoleucine, it was found that activity was lost completely. About 25% of the original activity could be restored by incubating fractions for 2 h at 30 °C with 1.0 mM dithiothreitol and 1.0 mM EDTA, but there was still a loss of activity when these reagents and isoleucine were added to the buffer mixture used for the chromatography (Table II). Considerable restoration of the activity of the resulting fractions could be

TABLE II

EFFECT OF PYRIDOXAL PHOSPHATE AND ALLOTHREONINE ON THE ACTIVITY OF THREONINE DEHYDRATASE

Threonine dehydratase (235 units, 33 units/mg) was chromatographed at 4 °C on a column (1.5 cm × 100 cm) of Sephadex G-100, which had been equilibrated with 0.05 M *N*-ethylmorpholine–HCl (pH 8.0) containing dithiothreitol (1.0 mM), EDTA (0.1 mM) and L-isoleucine (0.12 mM). The total activity of threonine dehydratase in the fractions eluted from the column was 118 units. The three fractions with the highest specific activity were combined to yield enzyme with an activity of 74 units/mg. After dilution of samples (0.5 ml) with an equal volume of 0.05 M *N*-ethylmorpholine–HCl (pH 8.0) containing allothreonine and/or pyridoxal phosphate, they were incubated for 3 h at 30 °C.

Additions	*Specific activity (μmoles/h per mg protein)*
None	0
Allothreonine (10 mM)	10
Pyridoxal phosphate (20 μM)	0
Allothreonine *plus* pyridoxal phosphate	119

achieved by their incubation with allothreonine and pyridoxal phosphate, but not with either compound alone. Indeed, under the latter conditions, there was a marked decrease in enzymic activity (Table II). It has become clear that dithiothreitol, allothreonine and pyridoxal phosphate are all required to maintain a stable form of threonine dehydratase.

When the reaction velocity was determined under the standard assay conditions but with threonine at a concentration of 0.5 mM, the rate of product formation was linear for only about 10 min even though after 90 min less than 3% conversion had occurred. Further, when the data were used for a plot of log $a/(a - x)$ as a func-

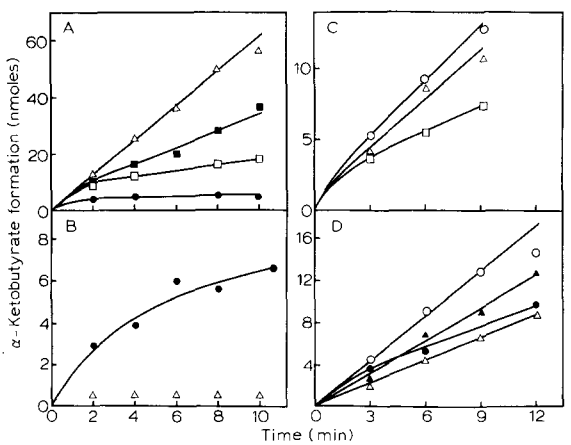


Fig. 4. Effect of buffer composition, pH, pyridoxal phosphate and KCl on the rate of α-ketobutyrate formation. Reaction mixtures for A and B contained 0.5 mM threonine, 8 μM pyridoxal phosphate, 4.3 μg of enzyme (208 units per mg) and 0.1 M Tricine–NaOH (A) or 0.1 M Tris–HCl (B). The buffer solutions were: A, pH 7.5 (●); pH 8.0 (□); pH 8.5 (■); pH 9.0 (△); B, pH 8.5 (△); pH 9.0 (●). Reaction mixtures for C and D contained 0.1 M TES–NaOH (pH 7.4), 0.84 mM threonine and 7.8 μg of enzyme (183 units per mg) as well as the following components: C, 0.1 M KCl and pyridoxal phosphate at concentrations of 0.04 μM (□); 2 μM (△); 10 μM (○). D, 10 μM pyridoxal phosphate and KCl at concentrations of zero (●); 0.05 M (○); 0.1 M (▲); 0.15 M (△).

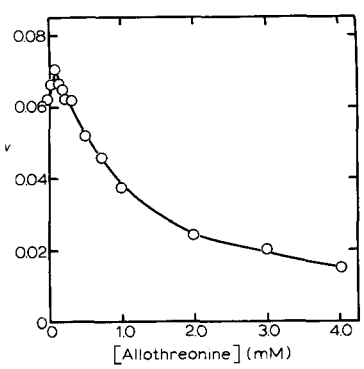
tion of time, where a represents the amount of threonine initially present in the reaction mixture, and x, the amount converted to products at time t, the resulting curve was non-linear. By contrast, a rapid fall off in the reaction rate was not observed with concentrations of threonine greater than 1.0 mM. Thus it has been concluded that the enzyme undergoes inactivation in the presence of relatively low substrate concentrations and such a conclusion is consistent with the finding that allothreonine is necessary for the stabilization of the enzyme during purification. Similar results have been obtained with the threonine dehydratase enzymes from *S. typhimurium*[9] and *Escherichia coli*[8]. The rate of enzyme inactivation at substrate concentrations less than 1.0 mM was also dependent on pH, composition of the buffer and the concentration of pyridoxal phosphate (Figs 4A–4C). The addition of KCl proved to be an effective means of preventing inactivation of the enzyme and of increasing its activity provided that the concentration did not exceed 0.1 M (Fig. 4D).

Activation and inhibition by allothreonine

Allothreonine, at concentrations below 0.15 mM, was found to activate threonine dehydratase when lower substrate concentrations were used, while higher concentrations of this threonine analogue were inhibitory (Fig. 5). The inhibition by allothreonine of the enzymes from *S. typhimurium*[29] and *E. coli*[30] has also been observed.

Inhibition by isoleucine

From the data of Fig. 6A, it is apparent that the inhibition of threonine dehydratase by isoleucine increases over a period of about 5 min before a constant velocity is attained. This effect, which is most marked when the concentration of isoleucine is greater than 50 μM and that of threonine is lower than 5 mM, contrasts with the observed linearity of the reaction in the absence of isoleucine (Fig. 6B). The result was not due to a limited non-enzymic reaction for there was no apparent formation of α-ketobutyrate when inactive enzyme (heated at 100 °C for 5 min) was tested



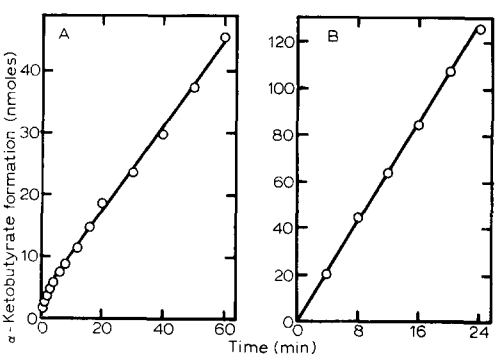


Fig. 5. Activation and inhibition of threonine dehydratase by allothreonine. Reaction mixtures contained 0.1 M TES–NaOH buffer (pH 7.4), 0.5 mM threonine, 2 μM pyridoxal phosphate, 1.74 μg of enzyme (1630 units per mg) and the indicated concentrations of allothreonine. Velocity is expressed as μmoles/h per μg of protein.

Fig. 6. Time course of the reaction in the presence of isoleucine (A) and in the absence of isoleucine (B). Reaction mixtures contained 0.1 M TES–NaOH buffer (pH 7.4), 0.1 M KCl, 3.5 mM threonine, 2 μM pyridoxal phosphate, 1.26 μg of enzyme (2900 units per mg) and 70 μM isoleucine (A).

under the same conditions. It also differed from the linear plots obtained in studies of the inhibition of the reaction by allothreonine. Therefore, it appears that the inhibition by isoleucine involves the slow conversion of an active form of the enzyme to one which is less active. Since the slope of the asymptotes of the curves obtained in the presence of 70 μM isoleucine (*cf.* Fig. 6A) varied as a linear function of the enzyme concentration, the conclusion was reached that the slope of the asymptote gives a true measure of the inhibited reaction velocity.

DISCUSSION

The results of the present work indicate clearly that dithiothreitol, pyridoxal phosphate and allothreonine are required to produce and maintain a stable, active form of threonine dehydratase from *R. spheroides*. Indeed, it was this finding that permitted further purification of the enzyme. In this connection it is of interest to note that the conditions for the stabilization of the *R. spheroides* threonine dehydratase differ from those which are necessary to stabilize the enzyme from other microorganisms. Thus the *S. typhimurium* enzyme is stable in the presence of dithiothreitol and isoleucine[2] while that from *B. subtilis* requires only mercaptoethanol and pyridoxal phosphate for stabilization[3].

Because threonine can react with pyridoxal phosphate bound to threonine dehydratase to form a Schiff base[31] and as the enzyme from *R. spheroides* loses activity unless high concentrations of threonine are present, it appears that it is the formation of a Schiff base that confers stability on the enzyme. The same rationale can be invoked to explain the stabilizing effect of allothreonine in the presence of pyridoxal phosphate, especially as allothreonine acts as a linear competitive inhibitor[32] and therefore presumably reacts with the enzyme in the same manner as does threonine. Since pyridoxal phosphate alone is incapable of producing a stable form of enzyme, it follows that both the free enzyme and the enzyme–pyridoxal phosphate complex are unstable. Consequently, the stability of threonine dehydratase under any set of conditions will be determined by the proportion of total enzyme that is present as a ternary complex involving pyridoxal phosphate and threonine (or allothreonine). On the other hand, the decreased loss of enzymic activity at pH 9.0 or in the presence of KCl (Fig. 4) is probably due to the formation of different ionic species of enzyme which are inherently more stable.

The time-dependent development of the full inhibition of the *R. spheroides* enzyme by isoleucine (Fig. 6A) is consistent with the occurrence of a relatively slow isomerisation of the complex formed when isoleucine reacts at a modifier site. Since it appears that the maximum velocity of the reaction is not affected by isoleucine[32], it may be concluded that the lower activity of the resulting isomeric form of enzyme is due to a reduction in its ability to bind threonine rather than to a decrease in catalytic activity. Threonine dehydratase from *B. subtilis* also exhibits a time lag before a steady-state inhibited velocity is attained after the addition of enzyme in the presence of isoleucine[3,4]. Evidence has been presented to show this effect is related to the ability of isoleucine to cause the formation of a modified form of enzyme whose lower activity is retained on removal of isoleucine and which, in the absence of isoleucine, can revert to the fully active form either spontaneously or more rapidly on the addition of threonine[4].

The inhibition obtained at higher concentrations of allothreonine (Fig. 5) is in accord with the finding that this compound acts as a linear competitive inhibitor[32]. But lower concentrations of such an inhibitor would not be expected to cause activation under conditions where the concentration of substrate is also relatively low. The result therefore suggests that the *R. spheroides* enzyme possesses multiple interdependent active sites so that allothreonine can occupy one (or more) of these and either enhance the binding of threonine at unoccupied sites or increase the catalytic activity of the resulting complex(es). Alternatively, allothreonine which bears structural resemblences to isoleucine and valine, could exert its activating effect by combining at the modifier site which is considered to be specific for isoleucine and valine. The data are not sufficiently extensive to distinguish between these two possibilities. However, in either case, increasing concentrations of allothreonine would eliminate the activation because of the increasing formation of dead-end enzyme–allothreonine complexes.

ACKNOWLEDGEMENTS

The authors are grateful to Miss M. Reed for skilful technical assistance. G.J.B. thanks the Australian Government for the award of an Australian Commonwealth Postgraduate Scholarship.

REFERENCES

1 H. E. Umbarger, in B. L. Horecker and E. R. Stadtman, *Current Topics in Cellular Regulation*, Vol. I, Academic Press, New York, 1969, p. 57.
2 R. O. Burns and M. H. Zarlengo, *J. Biol. Chem.*, 243 (1968) 178.
3 G. W. Hatfield and H. E. Umbarger, *J. Biol. Chem.*, 245 (1970) 1742.
4 G. W. Hatfield, W. J. Ray and H. E. Umbarger, *J. Biol. Chem.*, 245 (1970) 1748.
5 P. Datta, *J. Biol. Chem.*, 241 (1966) 5836.
6 H. de Robichon-Szulmajster and P. T. Magee, *Eur. J. Biochem.*, 3 (1968) 492.
7 C. Leitzmann and R. W. Bernlohr, *Biochim. Biophys. Acta*, 151 (1968) 449.
8 W. M. Harding, *Arch. Biochem. Biophys.*, 129 (1969) 57.
9 C. Cennamo and D. Carretti, *Biochim. Biophys. Acta*, 122 (1966) 371.
10 P. Datta, *Science*, 165 (1969) 556.
11 G. N. Cohen, R .Y. Stanier and G. Le Bras, *J. Bacteriol.*, 99 (1969) 791.
12 G. J. Barritt, *J. Bacteriol.*, 105 (1971) 718.
13 J. F. Morrison, W. J. O'Sullivan and A. G. Ogston, *Biochim. Biophys. Acta*, 52 (1961) 82.
14 J. Lascelles, *Biochem. J.*, 62 (1956) 78.
15 E. A. Noltmann, C. J. Gubler and S. A. Kuby, *J. Biol. Chem.*, 236 (1961) 1225.
16 E. Layne, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 450.
17 H. M. Kalckar, *J. Biol. Chem.*, 167 (1947) 461.
18 M. Orr, Ph. D. Thesis, Australian National University, 1969.
19 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
20 I. H. Fine and L. A. Costello, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. VI, Academic Press, New York, 1963, p. 958.
21 P. Andrews, *Biochem. J.*, 96 (1965) 595.
22 B. Chance, in D. Glick, *Methods of Biochemical Analysis*, Vol. I, Interscience, New York, 1954, p. 408.
23 E. Racker, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. I, Academic press, New York, 1955, p. 500.
24 B. Chance and A. C. Maehly, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 764.
25 L. Noda, S. Kuby and H. Lardy, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. II, Academic Press, New York, 1955, p. 605.
26 R. G. Martin and B. N. Ames, *J. Biol. Chem.*, 236 (1961) 1372.

27 T. Bucher, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. I, Academic Press, New York, 1955, p. 427.
28 J. H. R. Kagi and B. L. Vallee, *J. Biol. Chem.*, 235 (1960) 3188.
29 P. Maeba and B. D. Sanwal, *Biochemistry*, 5 (1966) 525.
30 J-P. Changeux, *Cold Spring Harbor Symp. Quant. Biol.*, 28 (1963) 497.
31 A. T. Phillips and W. A. Wood, *J. Biol. Chem.*, 240 (1965) 4703.
32 G. J. Barritt and J. F. Morrison, *Biochim. Biophys. Acta*, 284 (1972) 521.